GeneChip® 3′ IVT Express Kit User Manual

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Use of the GeneChip® 3′ IVT Express Kit in accordance with these instructions in preparing targets for GeneChip® probe arrays may be covered by U.S. patent numbers 5,545,522; 5,716,785; 5,891,636 and 6,291,170. Those who do not use this kit to prepare targets for GeneChip® probe arrays may be required to obtain a license under these patents. Products may be covered by U.S. Patent No. 6,864,059.

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Chapter 1

OVERVIEW

Introduction

The GeneChip® 3' IVT Express Kit is the latest technology in RNA target preparation for microarray expression analysis. This kit features:

- Low RNA input requirements- from as little as 50ng of total RNA for a single round of amplification
- Streamlined workflow, with the option to decrease target labeling time to a single day with appropriate inputs of total RNA
- Master mixes, consumables included and a simple protocol for ease of use, convenience and a high rate of success
- A complete kit that includes Poly-A RNA controls and hybridization controls
- Magnetic-bead aRNA purification for high recovery and ease of use.

The kit is based upon linear RNA amplification and employs T7 *in vitro* transcription technology. Also known as the Eberwine or reverse transcription-IVT (RT-IVT) method, this process is considered the gold standard for target preparation for gene expression analysis. RT-IVT was experimentally validated using TaqMan® RT-PCR (MAQC Consortium *et. al*, 2006).

In the GeneChip® 3' IVT Express Protocol total RNA undergoes reverse transcription to synthesize first-strand cDNA. This cDNA is then converted into a double-stranded DNA template for transcription. *In vitro* transcription synthesizes aRNA and incorporates a biotin-congugated nucleotide (cRNA is also known as amplified RNA or aRNA). The aRNA is then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate. Fragmentation of the biotin-labeled aRNA prepares the sample for hybridization onto GeneChip 3' expression arrays.

Assay Overview

The GeneChip 3’ IVT Express Kit aRNA amplification procedure is depicted in Figure 1.1.

- **Reverse Transcription to Synthesize First-Strand cDNA** is primed with T7 oligo(dT) primer to synthesize cDNA containing a T7 promoter sequence.
- **Second-Strand cDNA Synthesis** converts the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. The reaction employs DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA.
- ***In Vitro* Transcription to Synthesize Biotin-Modified aRNA with IVT Labeling Master Mix** generates multiple copies of biotin-modified aRNA from the double-stranded cDNA templates; this is the amplification step.
- **aRNA Purification** removes unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the biotin-modified aRNA.
- **Fragmentation** of the labeled aRNA prepares the target for hybridization to GeneChip® 3’ expression arrays.

Control RNA

Use the included Control RNA to familiarize yourself with the GeneChip 3’ IVT Express Kit RNA Amplification procedure. Instructions for the positive control reaction are provided in Appendix A, *Troubleshooting* on page 31.

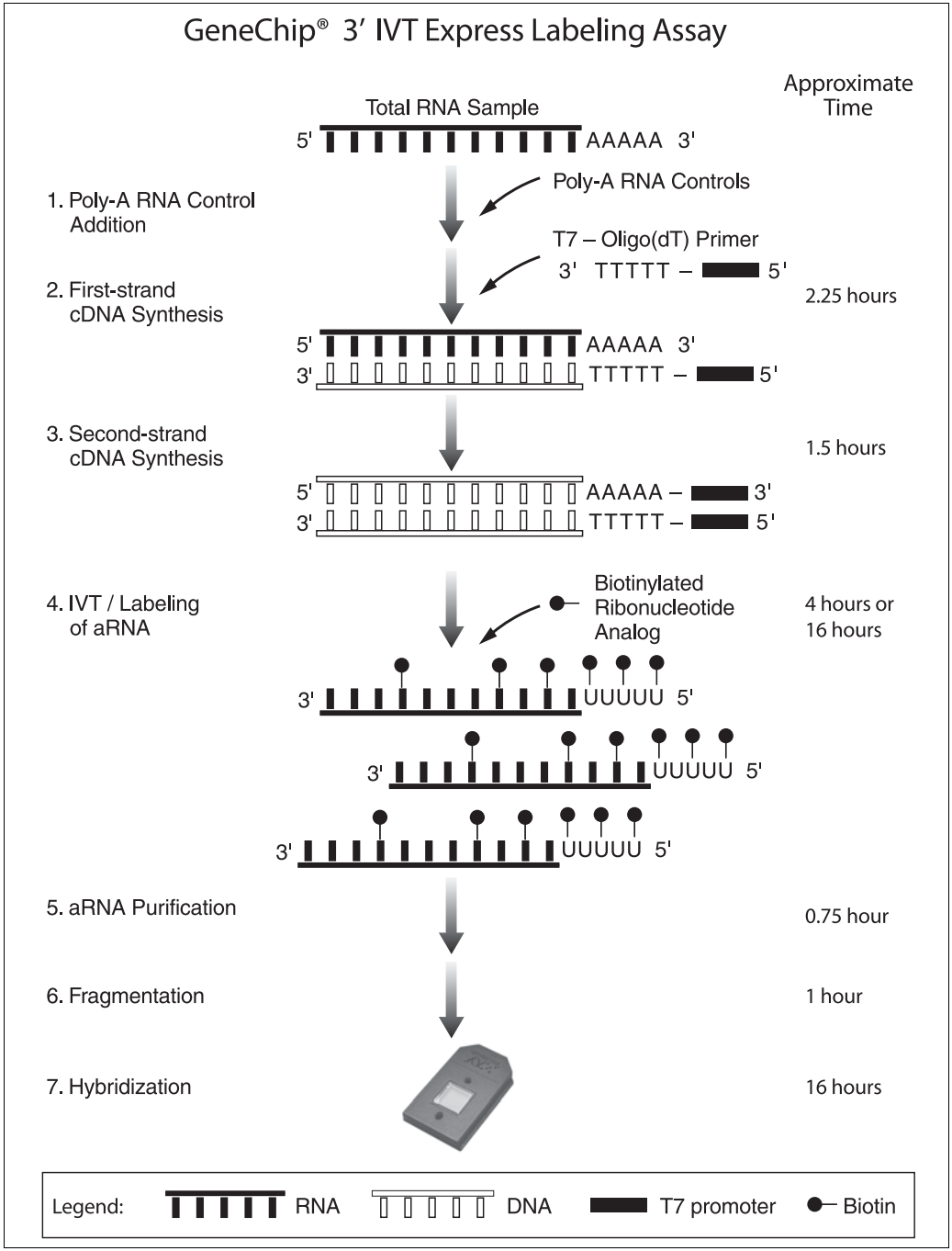


Figure 1.1 Overview of the GeneChip® 3′ IVT Express Kit Labeling Assay

Kit Contents and Storage Conditions

Table 1.1 GeneChip® 3′ IVT Express Kit Components and Storage Conditions

Component	Vol/Qnty 10 rxn	Vol/Qnty 30 rxn	Storage
BOX 1 of 2			
aRNA Binding Buffer Concentrate	600 µL	1.8 mL	room temp
RNA Binding Beads	120 µL	360 µL	2-8 °C*
aRNA Wash Solution Concentrate (*Add 8 mL 100% ethanol before use, as shown on the label)*	10 mL	10 mL	room temp
aRNA Elution Solution	5 mL	5 mL	room temp
Nuclease-free Water	10 mL	10 mL	room temp
5X Array Fragmentation Buffer	1 mL	1 mL	room temp
8-Strip PCR Tubes & Caps (0.2 mL)	10 ea.	20 ea.	room temp
U-Bottom Plate	1 ea.	2 ea.	room temp
Reservoir	1 ea.	1 ea.	room temp
BOX 2 of 2			
First-Strand Enzyme Mix	11 µL	33 µL	–20 °C
First-Strand Buffer Mix	44 µL	132 µL	–20 °C
Second-Strand Enzyme Mix	22 µL	66 µL	–20 °C
Second-Strand Buffer Mix	55 µL	165 µL	–20 °C
IVT Enzyme Mix	66 µL	198 µL	–20 °C
IVT Labeling Buffer	220 µL	660 µL	–20 °C
IVT Biotin Label	44 µL	132 µL	–20 °C
Control RNA (1 mg/mL HeLa total RNA)	10 µL	10 µL	–20 °C
Nuclease-free Water	1.75 mL	1.75 mL	–20 °C
Poly-A Control Stock	16 µL	16 µL	–20 °C
Poly-A Control Dilution Buffer	3.8 mL	3.8 mL	–20 °C
20X Hybridization Controls	450 µL	450 µL	–20 °C
Control Oligo B2	150 µL	150 µL	–20 °C

** Do not freeze.*

Materials

Required Reagents

Table 1.2 Reagents

Material	Source	P/N
GeneChip IVT Express Kits (See Table 1.1 for detailed kit information)	Affymetrix	901228 (10 Rxn) 901229 (30 Rxn)
GeneChip® Hybridization, Wash, and Stain Kit* containing: *Hybridization Module from Box 1* • Pre-Hybridization Mix • 2X Hybridization Mix • DMSO • Nuclease-free water *Stain Module from Box 1* • Stain Cocktail 1 • Stain Cocktail 2 • Array Holding Buffer *Wash Buffers A and B from Box 2* • Wash Buffer A (P/N 900721) • Wash Buffer B (P/N 900722)	Affymetrix	900720 (30 Rxn)
100% ethanol (ACS reagent grade)†	multiple	
Quant-iT™ RiboGreen® RNA Reagent (Optional)	Invitrogen	R11490

** For hybridization, washing and staining of the targets prepared using the GeneChip® 3′ IVT Express Kit onto GeneChip arrays, users should purchase the GeneChip® Hybridization, Wash, and Stain Kit.*

† Or equivalent.

Lab Equipment and Supplies

Table 1.3 Lab Equipment and Supplies

Material	Source	P/N
Lab Equipment and Supplies		
Thermal Cycler with heated Lid (capable of holding 0.2 mL tubes for reaction incubations)	multiple	
Vortex Mixer	multiple	
Microcentrifuge (with an adapter for the PCR strip-tubes or plates supplied with the kit)	multiple	
Magnetic Stand for 96-well plates	Ambion	#AM10050 (96-well Magnetic Stand) or #AM10027 (Magnetic Stand - 96)
Orbital shaker for 96-well plates (e.g., Barnstead/Lab-Line Titer Plate Shaker)	multiple	
Vacuum Centrifuge Concentrator (Optional)		
Spectrophotometer (e.g., NanoDrop® ND-8000 UV-Vis Spectrophotometer)	NanoDrop Technologies	ND-8000
Reagents and apparatus for preparation and electrophoresis of agarose gels (Optional)		
Miscellaneous Supplies		
Pipette for 0.1 to 2 µL*	Rainin	L-2
Pipette for 2 to 20 µL*	Rainin	L-20
Pipette for 20 to 200 µL*	Rainin	L-200
Pipette for 100 to 1000 µL*	Rainin	L-1000
Sterile-barrier, RNase-free Pipette Tips†	multiple	
Bioanalyzer	Agilent	
Non-stick RNase-free microfuge tubes, 0.5 mL	Ambion	N12350
Non-stick RNase-free microfuge tubes, 1.5 mL	Ambion	12450

** Or equivalent.*

† Tips must be pointed, not rounded, for efficient use with the probe arrays. Beveled pipette tips may cause damage to the array septa and cause leakage.

Instruments

Table 1.4 Instruments

Instruments	Manufacturer	P/N
GeneChip® Hybridization Oven 640	Affymetrix	800138 (110 v) 800139 (220 v)
GeneChip® Fluidics Station 450	Affymetrix	00-0079
GeneChip® Scanner 3000 (or higher)	Affymetrix	See www.affymetrix.com

Chapter 2

aRNA AMPLIFICATION PROTOCOL

Important Parameters for Successful Amplification

Input RNA Quantity and IVT Reaction Incubation Time

NOTE: **The RNA volume must be ≤ 5 µL (≤ 3 µL if poly-A RNA controls are used).**

Consider both the type and amount of sample RNA available and the amount of aRNA needed for your analysis when planning experiments using the GeneChip® 3' IVT Express Kit. Because mRNA content varies significantly with tissue type, the optimal amount of total RNA input and IVT incubation time should be determined empirically for each experimental system. The recommended input RNA amounts listed in Table 2.1 are based on using total RNA from HeLa cells; use these recommendations as a starting point. Table 2.2 shows the corresponding recommended IVT incubation times.

Table 2.1 Input RNA Limits

Recommendations	Amount
Recommended	100 ng
Minimum	50 ng
Maximum	500 ng

Table 2.2 Recommended IVT Incubation Times

RNA Amount	IVT Incubation Time
50–250 ng	16 hours
100–500 ng	4 hours

RNA Purity

RNA quality is the single most important factor affecting how efficiently an RNA sample will be amplified using GeneChip® 3' IVT Express Kit. RNA samples should be free of contaminating proteins, DNA, and other cellular material as well as phenol, ethanol, and salts associated with RNA isolation procedures. Impurities can lower the efficiency of reverse transcription and subsequently reduce the level of amplification. An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The ratio of A_{260} to A_{280} values should fall in the range of 1.7–2.1. RNA must be suspended in high quality water, TE (10 mM Tris-HCl, 1 mM EDTA.

RNA Integrity

The integrity of the RNA sample, or the proportion that is full length, is another important component of RNA quality. Reverse transcribing partially degraded mRNAs will generate cDNAs that may lack portions of the transcripts that are interrogated by probes on the array. RNA integrity can be evaluated by microfluidic analysis using the Agilent 2100 bioanalyzer with an RNA LabChip® Kit. Primarily full-length RNA will exhibit a ratio of 28S to 18S rRNA bands that approaches 2:1. Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity. The RIN, a metric developed by Agilent, includes information from both the rRNA bands and outside the rRNA peaks (potential degradation products) to provide a picture of RNA degradation states. Search for "RIN" at the following web site for further information: www.chem.agilent.com.

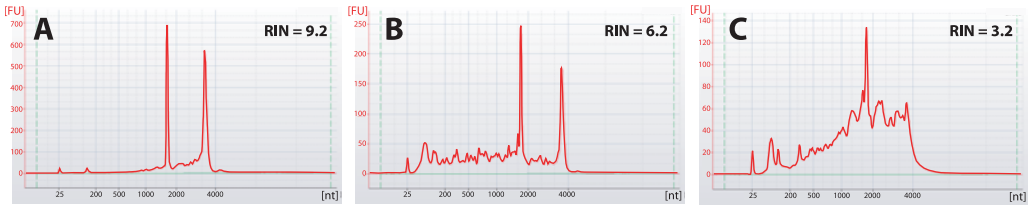


Figure 2.1 Example Agilent Bioanalyzer Electropherograms from three different total RNAs of varying integrity. Panel [A] represents a highly intact total RNA (RIN = 9.2), panel [B] represents a moderately intact total RNA (RIN = 6.2), and panel [C] represents a degraded total RNA sample (RIN = 3.2).

NOTE: Total RNAs with lower RIN values may require increased input amounts to generate enough aRNA for hybridization to an array.

Denaturing agarose gel electrophoresis and nucleic acid staining can also be used to separate and visualize the major rRNA species. When the RNA resolves into discrete rRNA bands (i.e., no significant smearing below each band), with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band, then the mRNA in the sample is likely to be mostly full-length. The primary drawback to gel electrophoresis is that it requires microgram amounts of RNA.

Other Important Parameters

- **Keep reaction incubation times precise and consistent**:
  The incubation times for the enzymatic reactions in the protocol were optimized in conjunction with the kit reagents for maximum yield in each step—adhere to them closely.
- **Use master mixes**:
  We strongly recommend preparing master mixes for each step of the GeneChip 3' IVT Express procedure. This reduces the effects of pipetting error, saves time, and improves reproducibility. The fill volumes in the kit allow for a ~5% overage when making master mixes.
- **Mix each kit component before use**:
  - Mix enzyme solutions by gently flicking the tube a few times before adding them to master mixes.
  - Thaw frozen reagents completely at room temperature, then mix thoroughly by vortexing, and place on ice.
- **Incubate reactions in a calibrated thermal cycler**:
  - We do not recommend using ordinary laboratory heat blocks, water baths, or hybridization ovens for any of the reaction incubations.
  - The procedure is very sensitive to temperature; therefore use a thermal cycler that has been calibrated according to the manufacturer's recommended schedule. Variable or inaccurate incubation temperatures can negatively impact aRNA synthesis.
  - Heated lids: It is important that condensation does not form in the tubes during any of the incubations, because it would change the reaction composition and can greatly reduce yield. If possible, set the lid temperature to match the block temperature. Otherwise, incubate all reactions with the heated lid on (~100 °C).
- **Maintain procedural consistency**:
  Procedural consistency is very important for amplification experiments. Consider implementing a detailed procedural plan that will be used by everyone in the lab to maintain consistency. This type of plan will minimize variation due to subtle procedural differences that can influence RNA amplification and may complicate gene expression studies. The plan should include basic information such as the method of RNA isolation, the amount of RNA to use in the procedure, and how long to incubate the IVT reaction. It should also address specifics that are not often included in protocols such as which tubes and thermal cycler to use for each step in the process. Finally, develop a consistent work flow. For example standardize stopping points in the method. The idea is to standardize all of the variables discussed in this section of the Instruction Manual and carefully follow all the protocol steps in order to maximize amplification consistency among samples.

Equipment and Reagent Preparation

Prepare aRNA Wash Solution

- Add 100% ethanol (ACS reagent grade or equivalent) to the bottle labeled aRNA Wash Solution Concentrate, as indicated on the label.
- Mix well and mark the label to indicate that the ethanol was added. This solution will be referred to as *aRNA Wash Solution* in these instructions. Store at room temperature.

Program the Thermal Cycler

Incubate all reactions in a thermal cycler. We find it convenient to set up the thermal cycler programs for each incubation before starting the procedure. The specifications for each incubation are shown in Table 2.3.

Table 2.3 Thermal Cycler Programs for RNA Amplification

Program (or Method)			
First-Strand cDNA Synthesis	42 °C for 2 hrs	4 °C indefinite hold	
Second-Strand cDNA Synthesis	16 °C for 1 hr	65 °C for 10 min	4 °C indefinite hold
IVT	40 °C for 4 or 16 hrs	4 °C indefinite hold	
Fragmentation	94 °C for 35 min	4 °C indefinite hold	

Preparation of Poly-A RNA Controls

Components of the GeneChip® IVT Express Kit, box 2, are used for this step.

Designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labeling process, a set of poly-A RNA controls is supplied in the GeneChip® 3’ IVT Express Kit.

Each eukaryotic GeneChip probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys, phe, thr*, and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are premixed at staggered concentrations. The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized below in Table 2.4.

Table 2.4 Final Concentrations of Poly-A RNA Controls when added to total RNA Samples

Poly-A RNA Spike	Final Concentration (ratio of copy number)
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

The controls are then amplified and labeled together with the total RNA samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples.

The **Poly-A RNA Control Stock** and **Poly-A Control Dil Buffer** are provided in the GeneChip IVT Express Kit to prepare the appropriate serial dilutions based on Table 2.5. This is a guideline when 50, 100, 250 or 500 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

IMPORTANT: Use non-stick RNase-free microfuge tubes to prepare all of the dilutions (not included).

Table 2.5 Serial Dilution of Poly-A RNA Control Stock

Total RNA Input Amount	Serial Dilutions				Volume of 4th dilution to add to total RNA
	First Dilution	Second Dilution	Third Dilution	Fourth Dilution	
50 ng	1:20	1:50	1:50	1:20	2 µL
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

Recommendation: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

For example, to prepare the poly-A RNA dilutions for 100 ng of total RNA:

1. Add 2 µL of the **Poly-A Control Stock** to 38 µL of **Poly-A Control Dil Buffer** for the first dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the First Dilution to 98 µL of **Poly-A Control Dil Buffer** to prepare the Second Dilution (1:50)
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µL of the Second Dilution to 98 µL of **Poly-A Control Dil Buffer** to prepare the Third Dilution (1:50)
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of the Third Dilution to 18 µL of **Poly-A Control Dil Buffer** to prepare the Fourth Dilution (1:10)
8. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
9. Add 2 µL of this Fourth Dilution to 100 ng of total RNA.

NOTE: The first dilution of the poly-A RNA controls can be stored up to six weeks in a non-frost-free freezer at –20 ºC and frozen-thawed up to eight times.

Table 2.6 Total RNA/Poly-A RNA Control Mixture

Component	Volume
Total RNA Sample (50-500 ng)	variable
Diluted Poly-A RNA Controls (Fourth Dilution)	2 µL
Nuclease-free Water	variable
Total Volume	5 µL

Reverse Transcription to Synthesize First-Strand cDNA

Components of the GeneChip® IVT Express Kit, box 2, are used for this step.

1. Assembly of **First-Strand Master Mix**.
   - **A.** Thaw first-strand synthesis reagents and place on ice.
   - **B.** On ice, assemble **First-Strand Master Mix** in a nuclease-free tube in the order listed in Table 2.7. Include ~ 5% overage to cover pipetting error.

Table 2.7 First-Strand Master Mix (for a single reaction)

Component	Amount
First-Strand Buffer Mix	4 µL
First-Strand Enzyme Mix	1 µL
Total volume	5 µL

   - **C.** Mix well by gently vortexing. Centrifuge briefly (~5 seconds) to collect the mix at the bottom of the tube.
   - **D.** Place the supplied PCR Tubes or Plate on ice and transfer 5 µL First-Strand Master Mix to individual tubes or wells.
2. Addition of Total RNA/poly-A Control Mixture.
   - **A.** Add 5 µL of the Total RNA/poly-A Control Mixture (Table 2.6) to each aliquot of First-Strand Master Mix for a final volume of 10 µL.
   - **B.** Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tube/plate and place on ice.
3. Incubation.
   - **A.** Incubate for 2 hours at 42 °C in a thermal cycler using the program for “First-Strand cDNA Synthesis” (Table 2.3 on page 12).
   - **B.** After the incubation, centrifuge briefly (~5 seconds) to collect the first-strand cDNA at the bottom of the tube/plate. Place the sample on ice and immediately proceed to Second-Strand cDNA synthesis (below).

Second-Strand cDNA Synthesis

Components of the GeneChip® IVT Express Kit, box 2, are used for this step.

1. Assembly of **Second-Strand Master Mix**.

   A. On ice, prepare a **Second-Strand Master Mix** in a nuclease-free tube in the order listed in Table 2.8. Prepare master mix for all the samples in the experiment, including ~ 5% overage to cover pipetting error.

Table 2.8 Second-Strand Master Mix (for a single reaction)

Component	Amount
Nuclease-free Water	13 µL
Second-Strand Buffer Mix	5 µL
Second-Strand Enzyme Mix	2 µL
Total Volume	20 µL

   B. Mix well by gently vortexing. Centrifuge briefly (~5 seconds) to collect the mix at the bottom of the tube and place on ice.

   C. Transfer 20 µL Second-Strand Master Mix to each (10 µL) cDNA sample. Mix thoroughly by gently vortexing or flicking the tube 3–4 times. Centrifuge briefly to collect the reaction at the bottom of the tube/plate and place on ice.

   D. Place the reaction in a 16 °C thermal cycler block. It is important to pre-cool the thermal cycler block to 16 °C because subjecting the reaction to temperatures >16° C will compromise aRNA yield.

2. Incubation.

   A. Incubate for 1 hour at 16 °C followed by 10 minutes at 65 °C in a thermal cycler using the program for “Second-Strand cDNA Synthesis” (Table 2.3 on page 12).

NOTE: Cover reactions with the heated lid of the thermal cycler even if its temperature cannot be adjusted to match the block temperature.

   B. After the incubation, centrifuge briefly (~5 seconds) to collect the double-stranded cDNA at the bottom of the tube/plate.

   C. Place on ice and immediately proceed to the IVT (below) or freeze at –20 °C.

TIP: STOPPING POINT Samples can be stored overnight at –20 °C at this point if desired.

In Vitro Transcription to Synthesize Labeled aRNA

Components of the GeneChip® IVT Express Kit, box 2, are used for this step.

1. Assembly of **IVT Master Mix**.

   A. At room temp, prepare an **IVT Master Mix** in a nuclease-free tube in the order listed in Table 2.9. Prepare master mix for all the samples in the experiment, including ~ 5% overage to cover pipetting error.

Table 2.9 IVT Master Mix (for a single reaction)

Component	Amount
IVT Biotin Label	4 µL
IVT Labeling Buffer	20 µL
IVT Enzyme Mix	6 µL
Total volume	30 µL

   B. Mix well by gently vortexing. Centrifuge briefly (~5 seconds) to collect the mix at the bottom of the tube and place on ice.

   C. Transfer 30 µL of **IVT Master Mix** to each (30 µL) double-stranded cDNA sample. Mix thoroughly by gently vortexing, and centrifuge briefly to collect the reaction at the bottom of the tube/plate.

   D. Once assembled, place the reaction in the thermal cycler block.

2. Incubation.

   Incubate the IVT reaction for 4 or 16 hours at 40 °C in a thermal cycler using the program for "IVT" (Table 2.3 on page 12). The recommended incubation time is based on the amount of input RNA and is shown in Table 2.10.

Table 2.10 Recommended IVT Incubation Times

RNA Amount	IVT Incubation Time
50–250 ng	16 hours
100–500 ng	4 hours

NOTE: Optimal RNA input amount and IVT incubation time are sample-type dependent and should be determined empirically. It is recommended to keep input amount and IVT incubation time consistent within a given experiment.

3. Place the aRNA on ice briefly or freeze immediately.

   Place the reaction on ice and proceed to the aRNA purification step (below) or immediately freeze at –20 °C for overnight storage.

TIP: STOPPING POINT. The aRNA can be stored overnight at –20 °C at this point, if desired.

aRNA Purification

Components of the GeneChip® IVT Express Kit, box 1, are used for this step.

After synthesis, the aRNA is purified to remove enzymes, salts, and unincorporated nucleotides. Photos of the aRNA purification process can be found in Appendix B on page 35.

If a plate shaker other than the recommended Lab-Line Titer Plate Shaker will be used, approximate shaking speeds for each step can be found in Appendix C, *Shaker Speeds* on page 37.

Before Beginning the aRNA Purification:

Preheat the aRNA Elution Solution to 50–60 °C for at least 10 minutes.

NOTE: Aliquot the appropriate amount of aRNA Elution Solution (50 µL per sample plus ~10% overage) to a separate 1.5 mL RNase-Free Tube (not included) to insure thorough pre-heating of the Elution Solution.

1. Preparation of **aRNA Binding Mix**.

IMPORTANT: Prepare only the amount needed for all samples in the experiment plus ~10% overage to cover pipetting error.

At room temperature, assemble **aRNA Binding Mix** in a nuclease-free tube for all the samples in the experiment following the instructions in Table 2.11.

Table 2.11 aRNA Binding Mix Preparation Instructions (for a single reaction)

Component	Amount
RNA Binding Beads*	10 µL
aRNA Binding Buffer Concentrate	50 µL

** Mix the RNA Binding Beads by vortexing before dispensing.*

2. Addition of aRNA Binding Mix.
   - **A.** Add 60 µL aRNA Binding Mix to each sample.
   - **B.** Transfer each sample to a well of a U-Bottom Plate.
   - **C.** Mix by pipetting up and down several times.
3. aRNA binding.
   - **A.** Add 120 µL 100% ethanol to each sample.
   - **B.** Mix by pipetting up and down several times.
   - **C.** *Gently* shake for ≥ 2 minutes to thoroughly mix (setting 4 on the Lab-Line Titer Plate Shaker). The aRNA in the sample will bind to the RNA Binding Beads during this incubation.
4. RNA Binding Beads capture.
   - **A.** Move the plate to a magnetic stand and capture the magnetic beads, for ~5 minutes. When capture is complete, the mixture becomes transparent and the RNA Binding Beads will form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand used and the amount of aRNA in your sample.

     NOTE: For maximum aRNA recovery, mix well and ensure that the mixture is transparent (all of the beads have been captured) before proceeding.

   - **B.** Carefully aspirate and discard the supernatant without disturbing the magnetic beads; then remove the plate from the magnetic stand.
5. Bead Washing

   IMPORTANT: Make sure that ethanol has been added to the bottle of aRNA Wash Solution Concentrate before using it.

   - **A.** Add 100 µL aRNA Wash Solution to each sample, and shake at *moderate* speed for 1 minute (setting 7 on the Lab-Line Titer Plate Shaker).

     NOTE: The RNA Binding Beads may not fully disperse during this step; this is expected and will not affect RNA purity or yield.

   - **B.** Move the plate to a magnetic stand and capture the RNA Binding Beads as in the previous step.
   - **C.** Carefully aspirate and discard the supernatant without disturbing the RNA Binding Beads and remove the plate from the magnetic stand.

D. Repeat Step A through Step C to wash a second time with 100 µL of aRNA Wash Solution.

E. Move the plate to a shaker and shake the plate dry *vigorously* for 1 minute to evaporate residual ethanol from the beads (setting 10 on the Lab-Line Titer Plate Shaker).

6. aRNA Elution

A. Elute the purified aRNA from the RNA Binding Beads by adding 50 µL preheated (50–60 °C) aRNA Elution Solution to each sample.

B. *Vigorously* shake the plate for 3 minutes (setting 10 on the Lab-Line Titer Plate Shaker). Then check to make sure the RNA Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed.

C. Move the plate to a magnetic stand, and capture the RNA Binding Beads.

D. Transfer the supernatant, which contains the eluted aRNA, to a nuclease-free PCR tube.

7. Store aRNA at ≤ –20 °C or place on ice and proceed with quantitation and fragmentation.

Purified aRNA can be stored at ≤ –20 °C for up to 1 year. As with any RNA preparation, the number of freeze-thaw cycles should be minimized to maintain aRNA integrity.

Chapter 3

EVALUATION AND FRAGMENTATION OF aRNA

aRNA Quantitation and Expected Yield

Assessing aRNA Yield by UV Absorbance

The concentration of an aRNA solution can be determined by measuring its absorbance at 260 nm. We recommend using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just measure 2 µL of the aRNA sample directly.

Alternatively, the aRNA concentration can be determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and reading the absorbance in a traditional spectrophotometer at 260 nm. Find the concentration in µg/mL using the equation shown below. (1 A_{260} = 40 µg RNA/mL)

Assessing aRNA Yield With RiboGreen®

A_{260} X dilution factor X 40 = µg RNA/mL

If a fluorometer or a fluorescence microplate reader is available, the RiboGreen fluorescence-based assay for RNA quantitation (Invitrogen) is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen.

Expected Yield

The aRNA yield will depend on the amount and quality of poly(A) RNA in the input total RNA. Since the proportion of poly(A) RNA in total RNA is affected by influences such as health of the organism and the organ from which it is isolated, aRNA yield from equal amounts of total RNA may vary considerably. Figure 3.1 shows yield data for aRNA produced with the kit from several different types of input RNA.

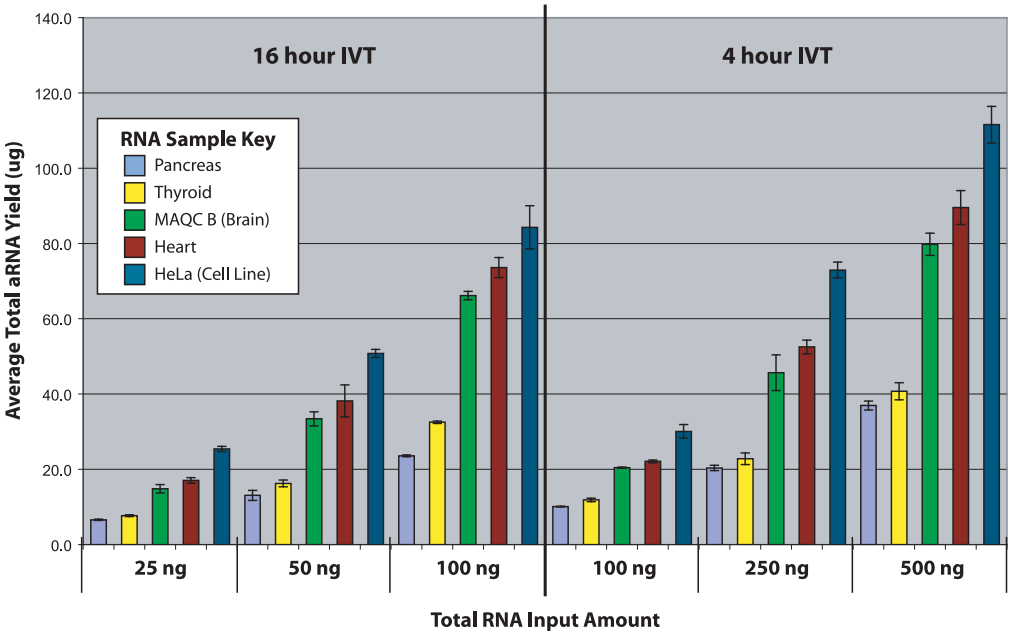


Figure 3.1 Average aRNA Yield from a variety of total RNA samples

(Optional) Concentrate the purified aRNA

If necessary, concentrate the aRNA by vacuum centrifugation. If the heater on the vacuum centrifuge has different settings, use medium or low. Check the progress of drying every 5–10 minutes, and remove the sample from the concentrator when it reaches the desired volume. Avoid drying aRNA samples to completion.

Analysis of aRNA Size

The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer with the Agilent RNA 6000 Nano Kit (P/N 5067-1511), or by conventional denaturing agarose gel analysis. The bioanalyzer can provide a fast and accurate size distribution profile of aRNA samples, but aRNA yield should be determined by UV absorbance or RiboGreen analysis. To analyze aRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified aRNA.

Expected aRNA Size

We recommend analyzing aRNA size distribution using an Agilent bioanalyzer and RNA 6000 Nano Kit loaded with 300 ng of aRNA per well. The expected aRNA profile is a distribution of sizes from 250–5500 nt with most of the aRNA between 600–1200 nt. Average aRNA size may vary slightly depending on RNA quality and total RNA input amount.

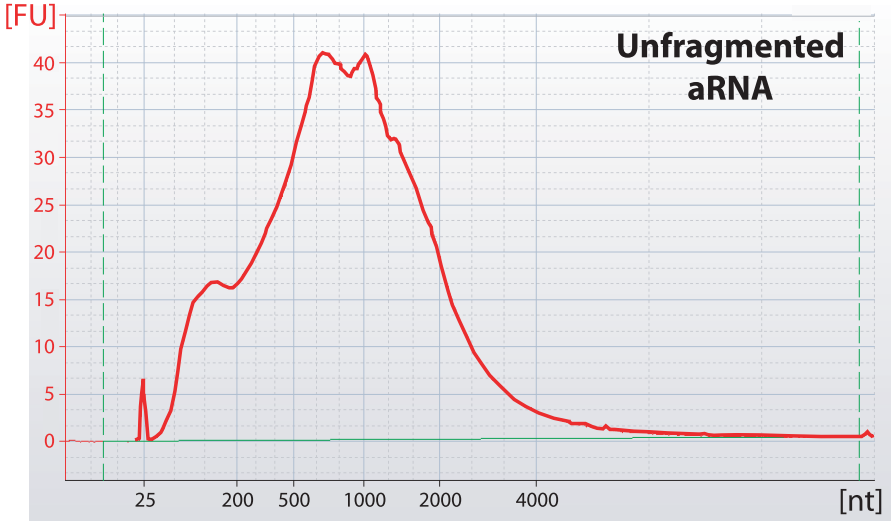


Figure 3.2 Example Agilent Bioanalyzer Electropherogram of un-fragmented aRNA generated from 50 ng of HeLa total RNA.

NOTE: Please refer to Chapter 4 for the amount of aRNA required for one array hybridization experiment. The amount varies depending on the array format. Please refer to the specific probe array package insert for information on the array format.

Fragmentation of Labeled aRNA

Components of the GeneChip® IVT Express Kit, box 1, are used for this step.

Fragmentation of aRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity.

Affymetrix recommends that the aRNA used in the fragmentation procedure be sufficiently concentrated to maintain a small volume during the procedure. This will minimize the amount of magnesium in the final hybridization cocktail. Fragment an appropriate amount of aRNA for hybridization cocktail preparation and gel analysis (aRNA amount depends on the format of the GeneChip probe array you are using).

1. Assemble the aRNA fragmentation mixture.

Table 3.1 Sample Fragmentation Reaction by Array Format*

Component	49/64 Format	100 Format	169/400 Format
aRNA	15 µg (1 to 32 µL)	12 µg (1 to 23.6 µL)	7.5 µg (1 to 16 µL)
5x Array Fragmentation Buffer	8 µL	6.4 µL	4 µL
Nuclease-free Water	Variable (up to 40 µL final volume)	Variable (up to 30 µL final volume)	Variable (up to 20 µL final volume)
Total Volume	40 µL	30 µL	20 µL

** Please refer to specific probe array package insert for information on array format.*

2. Fragmentation Reaction.
   - **A.** Incubate the fragmentation reaction at 94 ºC for 35 minutes.
   - **B.** Place the reaction on ice immediately after the incubation.
3. (Optional) Evaluate a sample of the reaction on a Bioanalyzer.

   Analyze the size of the fragmentation reaction products by running a 300 ng sample of the reaction on an Agilent bioanalyzer using an Agilent RNA 6000 Nano Kit. Figure 3.3 shows a typical result of such analysis. (Follow the manufacturer's instructions for this analysis.)

   The reaction should produce a distribution of 35–200 nt aRNA fragments with a peak at approximately 100-120 nt.

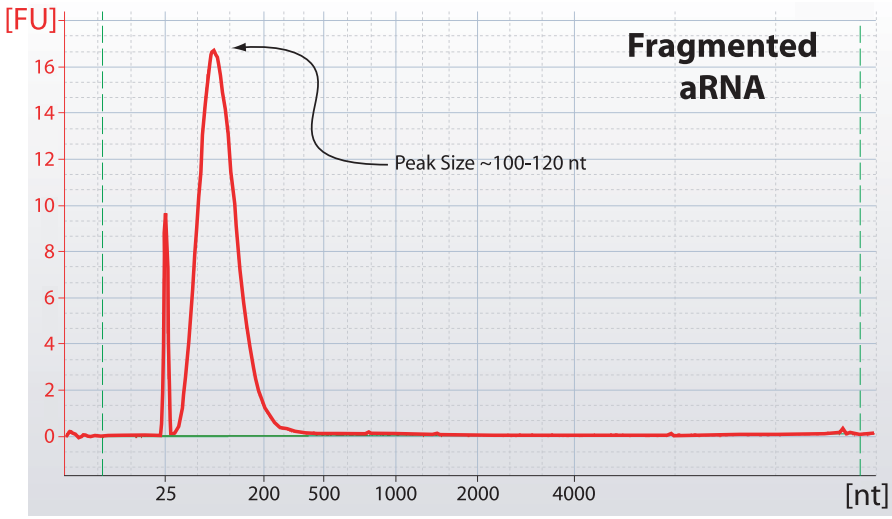


Figure 3.3 Example Agilent Bioanalyzer Electropherogram of fragmented aRNA.

4. Use fragmented aRNA immediately or store frozen.

Use the fragmented aRNA immediately or store undiluted, fragmented aRNA at –20 °C (or –70 °C for longer-term storage).

Chapter 4

HYBRIDIZATION

Overview

This chapter provides instruction for setting up cartridge array hybridizations using the **GeneChip® Hybridization, Wash, and Stain Kit, (30 rxns)**. For ordering information please refer to Table 1.2 on page 5.

Figure 4.1 Components of the GeneChip® Hybridization Wash and Stain Kit

Target Hybridization

Table 4.1 lists the necessary amount of aRNA required for the specific probe array format used. These preparations take into account that it is necessary to make extra hybridization cocktail due to a small loss of volume (10-20 µL) during each hybridization.

1. Mix the following for each target, scaling up volumes if necessary for hybridization to multiple probe arrays.

NOTE: DMSO will solidify when stored at 2-8 °C. Please ensure that the reagent is completely thawed prior to use. After the first use, it is recommended to store DMSO at room temperature.

Table 4.1 Hybridization Cocktail for Single Probe Array*

Component	*Array Format*			Final Dilution
	49 (Standard) / 64 Format	100 (Midi)	169 (Mini) / 400 (Micro)	
Fragmented and Labeled aRNA	12.5 µg (33.3 µL)	10 µg (26.7 µL)	5 µg (13.3 µL)	0.05 µg/ µL
Control Oligonucleotide B2 (3 nM)	4.2 µL	3.3 µL	1.7 µL	50 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	12.5 µL	10 µL	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	125 µL	100 µL	50 µL	1X
DMSO	25 µL	20 µL	10 µL	10%
Nuclease-free Water	50 µL	40 µL	20 µL	
Total Volume	250 µL	200 µL	100 µL	

** Please refer to specific probe array package insert for information on array format.*

IMPORTANT: It is imperative that frozen stocks of 20X GeneChip Eukaryotic Hybridization Controls are heated to 65 °C for 5 minutes to completely resuspend the aRNA before aliquoting.

2. Equilibrate probe array to room temperature immediately before use.

NOTE: It is important to allow the arrays to equilibrate to room temperature completely. Specifically, if the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which can lead to leaks.

3. Heat the hybridization cocktail to 99 °C for 5 minutes in a heat block.

4. Meanwhile, wet the array with an appropriate volume of Pre-Hybridization Mix (Table 4.2) by filling it through one of the septa.

Table 4.2 Probe Array Cartridge Volumes for Pre-Hybridization Mix and Hybridization Cocktail

Array	Volume
49 Format (Standard)	200 µL
64 Format	200 µL
100 Format (Midi)	130 µL
169 Format (Mini)	80 µL
400 Format (Micro)	80 µL

NOTE: Each array has two septa (see Figure 4.2 for location of the probe array septa). In order to fill the array, first vent the array chamber by inserting a clean, unused pipette tip into one of the septa; then insert the pipette tip of a micropipettor into the remaining septum to fill.

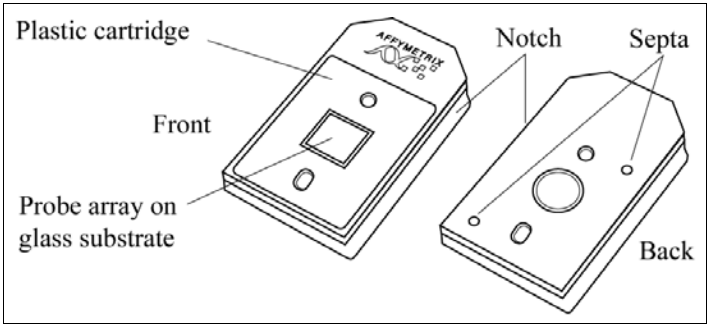


Figure 4.2 GeneChip® Probe Array

5. Incubate the probe array filled with Pre-Hybridization Mix at 45 °C for 10 minutes with rotation.
6. Transfer the hybridization cocktail that has been heated at 99 °C, in Step 3, to a 45 °C heat block for 5 minutes.
7. Spin the hybridization cocktail at maximum speed in a microcentrifuge for 5 minutes to collect any insoluble material from the hybridization mixture.
8. Remove the array from the hybridization oven. Vent the array with a clean pipette tip and extract the Pre-Hybridization Mix from the array with a micropipettor. Refill the array with the appropriate volume of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube (see Table 4.2).

9. Place probe array into the hybridization oven, set to 45 °C.
10. To avoid stress to the motor, load probe arrays in a balanced configuration around the axis. Rotate at 60 rpm.
11. Hybridize for 16 hours.

NOTE: During the latter part of the 16-hour hybridization prepare reagents for the washing and staining steps required immediately after completion of hybridization. For further instruction please refer to:

- ***Affymetrix® GeneChip® Fluidics Station 450/250 User Guide*** **(p/N 08-0092)**
- ***GeneChip® Expression Analysis Technical Manual with Specific Protocols for Using the GeneChip Hybridization, Wash and Stain Kit*** **(P/N 702232)**
- ***GeneChip® Expression Wash, Stain and Scan Manual for Cartridge Arrays*** **(P/N 702731)**

Appendix A

TROUBLESHOOTING

Positive Control Reaction

Control RNA Amplification Instructions

To verify that the process is working as expected, a Control RNA sample isolated from HeLa cells is provided with the kit.

1. Dilute 2 µL of the Control RNA into 18 µL of Nuclease-free Water.
2. Use 1 µL of the diluted Control RNA (100 ng); follow the protocol starting at *Reverse Transcription to Synthesize First-Strand cDNA* on page 15.
3. At *In Vitro Transcription to Synthesize Labeled aRNA* on page 17, use a 16 hour incubation for the IVT reaction.
4. Continue with the procedure for making biotin-modified aRNA through *aRNA Purification* on page 18.

Expected Results

- The positive control reaction should produce ≥ 50 µg of aRNA.
- The average size of the aRNA should be ~800 nucleotides.

Factors that Affect Both Positive Control and Experimental Samples

If the positive control reaction yield or amplification product size does not meet expectations, consider the following possible causes and troubleshooting suggestions. These suggestions also apply to problems with amplification of experimental RNA.

Incubation Temperature(s) Were Incorrect

The incubation temperatures are critical for effective RNA amplification. Use only properly calibrated thermal cyclers for the procedure.

Condensation formed in the tube during the reaction incubation(s)

Condensation occurs when the cap of the reaction vessel is cooler (e.g., room temperature) than the bottom of the tube. As little as 1–2 µL of condensate in an IVT reaction tube throws off the concentrations of the nucleotides and magnesium, which are crucial for good yield.

If you see condensation, check to make sure that the heated lid feature of the thermal cycler is working properly.

Nuclease Contamination

Using pipettes, tubes, or equipment that are contaminated with nucleases can cleave the RNA or DNA being generated at each step in the procedure. This will reduce the size of the aRNA products and decrease aRNA yield. Both RNases and DNases can be removed from surfaces using a RNase decontamination solution such as RNaseZap®.

Troubleshooting Low Yield and Small Average aRNA Size

Consider the following troubleshooting suggestions if the positive control reaction produced the expected results, but amplification of your experimental samples results in less or smaller (average <500 nt) aRNA than expected.

Lower Than Expected Input RNA Concentration

Take another A260 reading of your RNA sample or, if it is available, try using 100–250 ng of RNA in the amplification procedure.

impure RNA Samples

RNA samples with significant amounts of contaminating DNA, protein, phenol, ethanol, or salts are reverse transcribed poorly and subsequently generate less aRNA than pure RNA samples. Phenol extract and ethanol precipitate your RNA, or use a commercially available RNA cleanup kit to further purify your RNA before reverse transcription.

RNA Integrity is Compromised

RNA that is partially degraded generates cDNA that is relatively short. This will reduce the average size of the aRNA population and subsequently reduce the yield of aRNA. You can assess the integrity of an RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA (See *RNA Integrity* on page 10 for more information).

The mRNA Content of Your total RNA Sample is Lower Than Expected

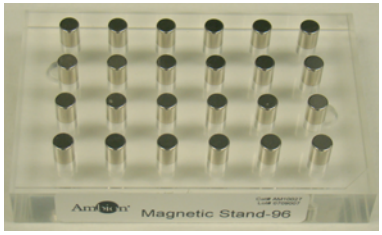
Different RNA samples contain different amounts of mRNA. In healthy cells, mRNA constitutes 1–10% of total cellular RNA (Johnson 1974, Sambrook and Russell 2001). The actual amount of mRNA depends on the cell type and the physiological state of the

sample. When calculating the amount of amplification, the starting mass of mRNA in a total RNA prep should always be considered within a range of 10–30 ng per µg of total RNA (assuming good RNA quality).

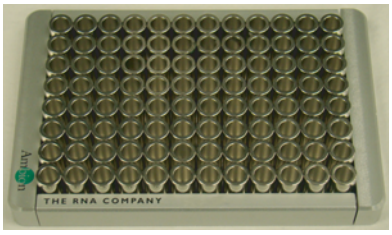
Appendix B

aRNA Purification Photos

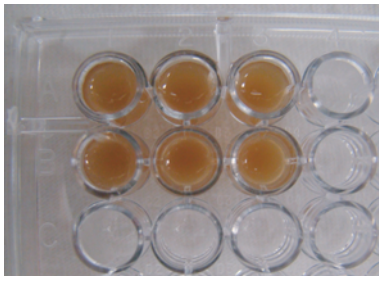
Ambion Magnetic Stand-96
(P/N #AM10027)



Ambion 96-well Magnetic-Ring Stand
(P/N #AM10050)

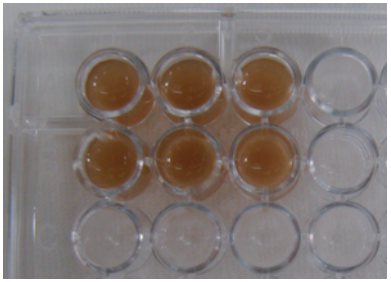


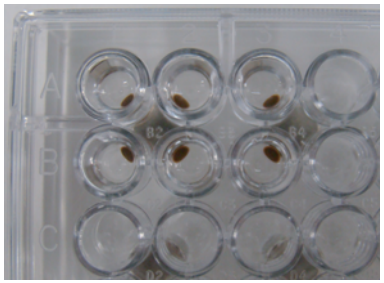
Magnetic Stand



aRNA Binding Step

After addition of ethanol and mixing





RNA Binding Beads Capture

After 5 minutes on magnetic stand

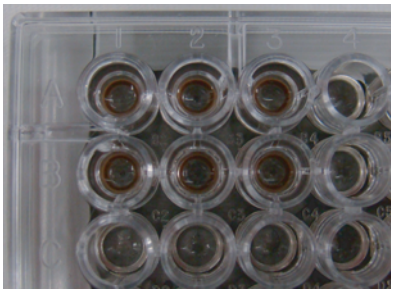


Figure B.1 Photos of aRNA Purification Step - figure 1 of 2

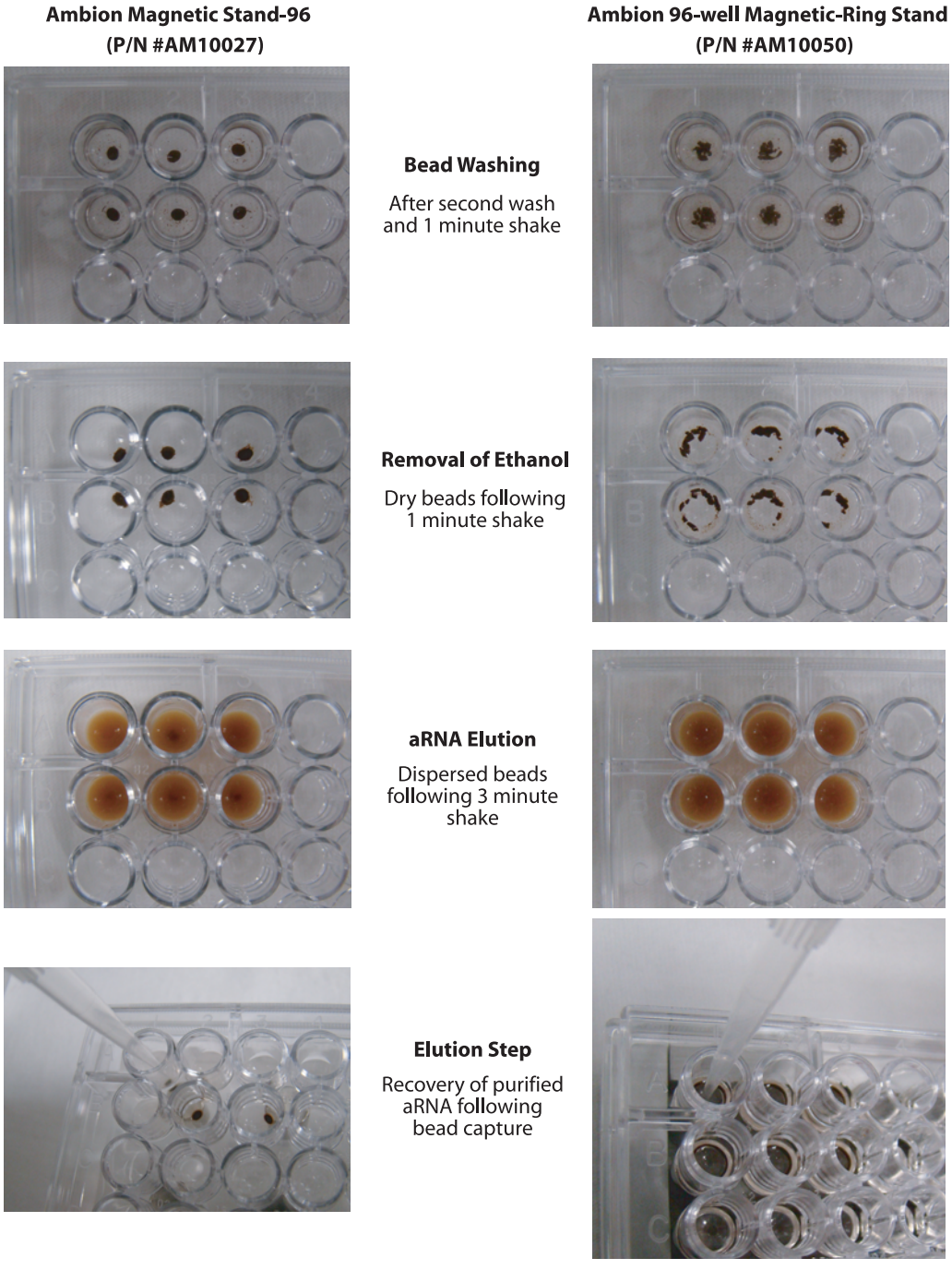


Figure B.2 Photos of aRNA Purification Step - figure 2 of 2

Appendix C

SHAKER SPEEDS

Table C.1 Plate Shaking Speeds

aRNA Purification Protocol Step	Shaking Speed	Approximate RPM Range	Recommended Speed Setting	
			Barnstead /Lab-Line Titer Plate Shaker (Model # 4625)	Boekel "Jitterbug" Plate Shaker (Model #130000
aRNA Binding	Gentle	300-500	4	1
Bead Washing	Moderate	700-900	7	4
Ethanol Removal	Vigorous	1000-1200	10	7
aRNA Elution	Vigorous	1000-1200	10	7